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(54) Title: METHOD OF PROMOTING HEPATIC CELL PROLIFERATION

(57) Abstract

The present invention provides methods, improved cell culture medium and kits for promoting hepatic cell proliferation and differentiation by growth in the presence of angiotensinogen, AI, AI anoalogues, and/or AI fragments and analogues thereof, AII analogues, AII fragments or analogues thereof, and/or AII AT_2 type 2 receptor agonists, either alone or in combination with other growth factors and cytokines.

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METHOD OF PROMOTING HEPATIC CELL PROLIFERATION

Cross Reference

This application is a continuation in part of U.S. Application Serial No. 60/074,104 filed February 9, 1998 and a continuation of U.S. Application Serial No. 60/108,412 filed November 13, 1998.

Field of the Invention

This present invention relates to methods, cell culture media, and kits for use in accelerating the proliferation of hepatic cells.

Background of the Invention

The liver is an epithelial organ that contains two major differentiated cell types: the hepatocyte and the bile ductule cell, which are thought to arise from a common hepatic stem cell. (Ponder, *FASEB J.* 10:673-684 (1996); hereby incorporated by reference in its entirety.) It is unclear what role the stem cell plays in normal postnatal liver growth, in the chronically regenerating liver, or in liver carcinogenesis.

Hepatic cells are typically quiescent in adults. (Thorgeirsson, *FASEB J.* 10:1249-1256 (1996); hereby incorporated by reference in its entirety.) However, both hepatocytes and bile ductule cells have the capacity to meet replacement demands caused by cellular loss, as exemplified by their ability to proliferate and restore liver mass after partial hepatectomy (PH). (*Id.*) The ability of liver tissue to regenerate has been known for some time and is controlled by cytokines of paracrine and/or autocrine origin which either stimulate or inhibit hepatic cell growth.

Hepatic stem cells, whose immediate progeny are referred to as oval cells or small epithelial cells (hereinafter referred to as 'SEC'), assume the burden of regenerative liver growth only after significant hepatocyte loss. In contrast to liver regeneration after PH, hepatic regeneration in acute liver failure (also known as fulminant hepatic failure) occurs by the proliferation and differentiation of hepatic stem cells. (Kay and Fausto, *Mol. Med. Today* 3:108-115 (1997); hereby incorporated by reference in its entirety.) Acute liver failure can be caused by several distinct diseases, including viral hepatitis, chemical injury (for example, caused by acetaminophen/paracetamol), Wilson's disease, and Reye's syndrome (*Id.*)

Oval cells or SEC arise initially in the bile epithelium after activation of hepatic stem cells following physiologically- or experimentally-induced liver damage (Alison et al., 1996; Thorgeirsson, 1996; Kay and Faust, 1997; and references therein). They are capable of differentiating into both bile epithelial cells and hepatocytes when given appropriate stimuli (Fausto, *Prog. Clin. Biol. Res.* 331:325 (1990); Sell, *Cancer Res.* 50:3811 (1990); Sigal et al., *Am. J. Physiol.* 263:G139 (1992); Thorgeirsson, *Am. J. Pathol.* 142:1331 (1993)).

Thus, the liver cannot be classified as a classical stem cell-fed tissue, like the epidermis, the intestinal epithelium, the hematopoietic system, or the bone marrow. Unlike other tissues capable of regeneration (e.g., skin and bone marrow), regeneration of the liver is not dependent upon small populations of stem cells or progenitor cells. However, large numbers of hepatic stem cells do appear when mature hepatocytes are inhibited from proliferation (Thorgeirsson, 1996; Sell, *Mod. Pathol.* 7:105 (1994); Fausto, et al., *Proc. Soc. Exp. Biol. Med.* 204:237 (1993)).

A number of acute and chronic conditions can lead to the damage of liver tissue. These include, but are not limited to, hepatocarcinoma, hepatitis infection, cirrhosis of the liver, partial hepatectomy, fulminant hepatic failure, hepatocyte transplantation, and liver transplantation. In most instances, the survival of patients with these disorders depends upon successful liver regeneration, which occurs through the proliferation of hepatocytes and/or hepatic stem cells.

Thus, there is a need in the art for methods that increase the proliferation of hepatic stem cells and hepatocytes. Such methods will provide clinical benefits in liver regeneration following resection of hepatocarcinomas, hepatitis infection, cirrhosis of the liver, partial hepatectomy, fulminant hepatic failure, hepatocyte transplantation, liver transplantation, and other hepatic disorders where rapid regeneration of the liver is desirable. Such methods are also useful in rapidly providing a large population of hepatic cells for use in cell therapy and for providing a large population of transfected hepatic cells for use in gene therapy.

Summary of the Invention

In one aspect, the present invention provides methods that promote hepatic cell proliferation by contacting the cells with angiotensinogen, angiotensin I (hereinafter referred to as 'AI'), AI analogues, and/or AI fragments and analogues thereof, angiotensin II (hereinafter referred to as 'AII') analogues, AII fragments or analogues thereof, and/or AII AT_2 type 2 receptor agonists, either alone or in combination with other growth factors and cytokines.

In another aspect of the present invention, an improved cell culture medium is provided for the proliferation of hepatic cells, wherein the improvement comprises

addition to the cell culture medium of an effective amount of angiotensinogen, AI, AI analogues, and/or AI fragments and analogues thereof, AII analogues, AII fragments or analogues thereof, and/or AII AT_2 type 2 receptor agonists.

In a further aspect, the present invention provides kits for the propagation of hepatic cells, wherein the kits comprise an effective amount of angiotensinogen, AI, AI analogues, and/or AI fragments and analogues thereof, AII analogues, AII fragments or analogues thereof, and/or AII AT_2 type 2 receptor agonists, and instructions for culturing the cells. Preferred embodiments of the kit further comprise cell culture growth medium, a sterile container, and an antibiotic supplement.

Brief Description of the Figures

Figure 1. Effect of AII and AII analogues on hepatocyte proliferation in vitro.

Detailed Description of the Preferred Embodiments

As defined herein, the term "hepatic cells" refers to hepatic stem cells that are poorly differentiated, demonstrate extensive proliferation, and have multiple differentiation options (Thorgeirsson, *FASEB J.* 10:1249 (1996)), as well as hepatocytes and bile ductule cells derived therefrom. Examples of hepatic stem cells include, but are not limited to, SEC cells, oval cells, as well as cultured WB-F344 cells and RLEΦ13 cells (Coleman et al., *Am. J. Pathol.* 151:353 (1997); Omori et al., *Hepatology* 26:720 (1997)). As defined herein, "proliferation" encompasses both cell self renewal and cellular proliferation with accompanying differentiation.

Unless otherwise indicated, the term "active agents" as used herein refers to the group of compounds comprising angiotensinogen, angiotensin I (AI), AI analogues, AI

fragments and analogues thereof, angiotensin II analogues, AII fragments or analogues thereof and AII AT_2 type 2 receptor agonists.

U.S. Patent No. 5,015,629 to DiZerega (the entire disclosure of which is hereby incorporated by reference) describes a method for increasing the rate of healing of wound tissue, comprising the application to such tissue of AII in an amount which is sufficient for said increase. The application of AII to wound tissue significantly increases the rate of wound healing, leading to a more rapid re-epithelialization and tissue repair. The term AII refers to an octapeptide present in humans and other species having the sequence Asp-Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:1]. The biological formation of angiotensin is initiated by the action of renin on the plasma substrate angiotensinogen (*Circulation Research* 60:786-790 (1987); Clouston et al., *Genomics* 2:240-248 (1988); Kageyama et al., *Biochemistry* 23:3603-3609; Ohkubo et al., *Proc. Natl. Acad. Sci.* 80:2196-2200 (1983); all references hereby incorporated in their entirety). The substance so formed is a decapeptide called angiotensin I (AI) which is converted to AII by the converting enzyme angiotensinase which removes the C-terminal His-Leu residues from AI, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu [SEQ ID NO:37]. AII is a known pressor agent and is commercially available.

Studies have shown that AII increases mitogenesis and chemotaxis in cultured cells that are involved in wound repair, and also increases their release of growth factors and extracellular matrices (diZerega, U.S. Patent No. 5,015,629; Dzau et. al., *J. Mol. Cell. Cardiol.* 21:S7 (Supp III) (1989); Berk et. al., *Hypertension* 13:305-14 (1989); Kawahara, et al., *BBRC* 150:52-9 (1988); Naftilan, et al., *J. Clin. Invest.* 83:1419-23 (1989); Taubman et al., *J. Biol. Chem* 264:526-530 (1989); Nakahara, et al., *BBRC* 184:811-8 (1992); Stouffer and Owens, *Circ. Res.* 70:820 (1992); Wolf, et

al., *Am. J. Pathol.* 140:95-107 (1992); Bell and Madri, *Am. J. Pathol.* 137:7-12 (1990)). In addition, AII was shown to be angiogenic in rabbit corneal eye and chick chorioallantoic membrane models (Fernandez, et al., *J. Lab. Clin. Med.* 105:141 (1985); LeNoble, et al., *Eur. J. Pharmacol.* 195:305-6 (1991)). Therefore, AII may accelerate wound repair through increased neovascularization, growth factor release, reepithelialization and/or production of extracellular matrix. The use of AII analogues and fragments, AT2 agonists, as well as AIII and AIII analogues and fragments in wound healing has also been described. (U.S. Patent No. 5,629,292; U.S. Patent No. 5,716,935; WO 96/39164; all references herein incorporated by reference in their entirety.)

Although AII has been shown to increase the proliferation of a number of cell types *in vitro*, it does not necessarily increase the proliferation of all cell types. The effect of AII on a given cell type has been hypothesized to be dependent, in part, upon the AII receptor subtypes the cell expresses (Shanugam et al., *Am. J. Physiol.* 268:F922-F930 (1995); Helin et al., *Annals of Medicine* 29:23-29 (1997); Bedecs et al., *Biochem J.* 325:449-454 (1997)). These studies have shown that AII receptor subtype expression is a dynamic process that changes during development, at least in some cell types (*Id.*)

Recently, AII has been identified as a co-mitogen for hepatocytes both in vitro and in vivo. (Huerta-Bahena et al., *Biochim. Biophys. Acta* 763:120 (1983); Booz et al., *Endocrinology* 130:3641 (1992); Hasewaga et al., *J. Cell Physiol.* 158:215 (1994); Kar et al., *J. Cell Physiol.* 168:141 (1992); Dajani et al., *J. Cell. Physiol.* 168:608 (1996)). However, it is not known whether angiotensinogen, AI, AI analogues, and/or AI fragments and analogues thereof, AII analogues, AII fragments or analogues

thereof, and/or AII AT_2 type 2 receptor agonists accelerate the proliferation of hepatic stem cells, hepatocytes, or bile ductule cells. For example, data suggests that the AII fragment AII(1-7) acts through a receptor(s) that is distinct from the AT1 and AT2 receptors which modulate AII activity. (Ferrario et al., J. Am. Soc. Nephrol. 9:1716-1722 (1998); Iyer et al., Hypertension 31:699-705 (1998); Freeman et al., Hypertension 28:104 (1996); Ambuhl et al., Brain Res. Bull. 35:289 (1994). Thus, the stimulatory effect of AII through activation of the AT1 or AT2 receptor does not shed any light on the potential stimulatory effect of AII(1-7).

A peptide agonist selective for the AT2 receptor (AII has 100 times higher affinity for AT2 than AT1) is p-aminophenylalanine6-AII ["(p-NH_2-Phe)6-AII)"], Asp-Arg-Val-Tyr-Ile-Xaa-Pro-Phe [SEQ ID NO.36] wherein Xaa is p-NH_2-Phe (Speth and Kim, *BBRC* 169:997-1006 (1990). This peptide gave binding characteristics comparable to AT2 antagonists in the experimental models tested (Catalioto, et al., *Eur. J. Pharmacol.* 256:93-97 (1994); Bryson, et al., *Eur. J. Pharmacol.* 225:119-127 (1992).

The effects of AII receptor and AII receptor antagonists have been examined in two experimental models of vascular injury and repair which suggest that both AII receptor subtypes (AT1 and AT2) play a role in wound healing (Janiak et al., *Hypertension* 20:737-45 (1992); Prescott, et al., *Am. J. Pathol.* 139:1291-1296 (1991); Kauffman, et al., *Life Sci.* 49:223-228 (1991); Viswanathan, et al., *Peptides* 13:783-786 (1992); Kimura, et al., *BBRC* 187:1083-1090 (1992)).

Many studies have focused upon AII(1-7) (AII residues 1-7) or other fragments of AII to evaluate their activity. AII(1-7) elicits some, but not the full range of effects elicited by AII (Pfeilschifter, et al., *Eur. J. Pharmacol.* 225:57-62 (1992); Jaiswal, et

al., *Hypertension* 19 (Supp. II):II-49-II-55 (1992); Edwards and Stack, *J. Pharmacol. Exper. Ther.* 266:506-510 (1993); Jaiswal, et al., *J. Pharmacol. Exper. Ther.* 265:664-673 (1991); Jaiswal, et al., *Hypertension* 17:1115-1120 (1991); Portsi, et a., *Br. J. Pharmacol.* 111:652-654 (1994)).

As hereinafter defined, a preferred class of AT2 agonists for use in accordance with the present invention comprises AI, AII, and AI or AII analogues or active fragments thereof having p-NH-Phe in a position corresponding to a position 6 of AII. In addition to peptide agents, various nonpeptidic agents (e.g., peptidomimetics) having the requisite AT2 agonist activity are further contemplated for use in accordance with the present invention.

The active AII analogues, fragments of AII and analogues thereof of particular interest in accordance with the present invention are characterized as comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general

formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group,

R^A is suitably selected from Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, Me^2Gly, Pro, Bet, Glu(NH_2), Gly, Asp(NH_2) and Suc,

R^B is suitably selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys,

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R^4 is selected from the group consisting of Tyr, $Tyr(PO_3)_2$, Thr, Ser, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group,

wherein the active agent is not AII.

Compounds falling within the category of AT2 agonists useful in the practice of the invention include the AII analogues set forth above subject to the restriction that R^6 is p-NH_2-Phe.

Particularly preferred combinations for R^A and R^B are Asp-Arg, Asp-Lys, Glu-Arg and Glu-Lys. Particularly preferred embodiments of this class include the following: AIII, Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:2]; AII(3-8), also known as des1-AIII or AIV, Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:3]; AII(1-7), Asp-Arg-Val-Tyr-Ile-His-Pro {SEQ ID NO:4]; AII(2-7). Arg-Val-Tyr-Ile-His-Pro [SEQ ID NO:5]; AII(3-7), Val-Tyr-Ile-His-Pro [SEQ ID NO:6]; AII(5-8), Ile-His-Pro-Phe [SEQ ID NO:7]; AII(1-6), Asp-Arg-Val-Tyr-Ile-His [SEQ ID NO:8]; AII(1-5), Asp-Arg-Val-Tyr-Ile [SEQ ID NO:9]; AII(1-4), Asp-Arg-Val-Tyr [SEQ ID NO:10]; and AII(1-3), Asp-Arg-Val [SEQ ID NO:11]. Other preferred embodiments include: Arg-norLeu-Tyr-Ile-His-Pro-Phe [SEQ ID NO:12] and Arg-Val-Tyr-norLeu-His-Pro-Phe [SEQ ID

NO:13]. Still another preferred embodiment encompassed within the scope of the invention is a peptide having the sequence Asp-Arg-Pro-Tyr-Ile-His-Pro-Phe [SEQ ID NO:31]. AII(6-8), His-Pro-Phe [SEQ ID NO:14] and AII(4-8), Tyr-Ile-His-Pro-Phe [SEQ ID NO:15] were also tested and found not to be effective.

A class of particularly preferred compounds in accordance with the present invention consists of those with the following general structure:

R1-Arg-R2-R3-R4-His-Pro-R5

wherein R1 is selected from the group consisting of H and Asp;

R2 is selected from the group consisting of Val and Pro;

R3 is selected from the group consisting of Tyr and $Tyr(PO_3)_2$;

R4 is selected from the group consisting of Ala, Ile, Leu, and norLeu; and

R5 is Phe, Ile, or is absent,

and wherein the active agent is not AII.

Particularly preferred embodiment of this class are selected from the group consisting of SEQ ID NO:4, SEQ ID NO:18, SEQ ID NO:26, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:34, and SEQ ID NO:38

Another class of compounds of particular interest in accordance with the present invention are those of the general formula II

$$R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^2 is selected from the group consisting of H, Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr$(PO_3)_2$, Thr, Ser, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr.

A particularly preferred subclass of the compounds of general formula II has the formula

R^2-R^3-Tyr-R^5-His-Pro-Phe [SEQ ID NO:16]

wherein R^2, R^3 and R^5 are as previously defined. Particularly preferred is a compound of the formula Arg-Val-Tyr-Ala-His-Pro-Phe [SEQ ID NO:38]. Other preferred compounds include peptides having the structures Arg-Val-Tyr-Gly-His-Pro-Phe [SEQ ID NO:17] and Arg-Val-Tyr-Ala-His-Pro-Phe [SEQ ID NO:18]. The fragment AII(4-8) was ineffective in repeated tests; this is believed to be due to the exposed tyrosine on the N-terminus.

In the above formulas, the standard three-letter abbreviations for amino acid residues are employed. In the absence of an indication to the contrary, the L-form of the amino acid is intended. Other residues are abbreviated as follows:

TABLE 1

Abbreviation for Amino Acids

Me^2Gly	N,N-dimethylglycyl

Bet	1-carboxy-N,N,N-trimethylmethanaminium hydroxide inner salt (betaine)
Suc	Succinyl
Phe(Br)	p-bromo-L-phenylalanyl
azaTyr	aza-α'-homo-L-tyrosyl
Acpc	1-aminocyclopentane carboxylic acid
Aib	2-aminoisobutyric acid
Sar	N-methylglycyl (sarcosine)

It has been suggested that AII and its analogues adopt either a *gamma* or a *beta* turn (Regoli, et al., *Pharmacological Reviews* 26:69 (1974)). In general, it is believed that neutral side chains in position R^3, R^5 and R^7 may be involved in maintaining the appropriate distance between active groups in positions R^4, R^6 and R^8 which are primarily responsible for binding to receptors and/or intrinsic activity. Hydrophobic side chains in positions R^3, R^5 and R^8 may also play an important role in the whole conformation of the peptide and/or contribute to the formation of a hypothetical hydrophobic pocket.

Appropriate side chains on the amino acid in position R^2 may contribute to affinity of the compounds for target receptors and/or play an important role in the conformation of the peptide. For this reason, Arg and Lys are particularly preferred as R^2.

For purposes of the present invention, it is believed that R^3 may be involved in the formation of linear or nonlinear hydrogen bonds with R^5 (in the gamma turn model) or R^6 (in the beta turn model). R^3 would also participate in the first turn in a beta antiparallel structure (which has also been proposed as a possible structure). In contrast

to other positions in general formula I, it appears that beta and gamma branching are equally effective in this position. Moreover, a single hydrogen bond may be sufficient to maintain a relatively stable conformation. Accordingly, R^3 may suitably be selected from Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr.

With respect to R^4, conformational analyses have suggested that the side chain in this position (as well as in R^3 and R^5) contribute to a hydrophobic cluster believed to be essential for occupation and stimulation of receptors. Thus, R^4 is preferably selected from Tyr, Thr, Tyr $(PO_3)_2$, homoSer, Ser and azaTyr. In this position, Tyr is particularly preferred as it may form a hydrogen bond with the receptor site capable of accepting a hydrogen from the phenolic hydroxyl (Regoli, et al. (1974), *supra*).

In position R^5, an amino acid with a β aliphatic or alicyclic chain is particularly desirable. Therefore, while Gly is suitable in position R^5, it is preferred that the amino acid in this position be selected from Ile, Ala, Leu, norLeu, Gly and Val.

In the AII analogues, fragments and analogues of fragments of particular interest in accordance with the present invention, R^6 is His, Arg or 6-NH_2-Phe. The unique properties of the imidazole ring of histidine (e.g., ionization at physiological pH, ability to act as proton donor or acceptor, aromatic character) are believed to contribute to its particular utility as R^6. For example, conformational models suggest that His may participate in hydrogen bond formation (in the *beta* model) or in the second turn of the antiparallel structure by influencing the orientation of R^7. Similarly, it is presently considered that R^7 should be Pro in order to provide the most desirable orientation of R^8. In position R^8, both a hydrophobic ring and an anionic carboxyl terminal appear to be particularly useful in binding of the analogues of interest to receptors; therefore, Tyr and especially Phe are preferred for purposes of the present invention.

Analogues of particular interest include the following:

TABLE 2: Angiotensin II Analogues

AII Analogue Name	Amino Acid Sequence	Sequence Identifier
Analogue 1	Asp-Arg-Val-Tyr-Val-His-Pro-Phe	SEQ ID NO: 19
Analogue 2	Asn-Arg-Val-Tyr-Val-His-Pro-Phe	SEQ ID NO: 20
Analogue 3	Ala-Pro-Gly-Asp-Arg-Ile-Tyr-Val-His-Pro-Phe	SEQ ID NO: 21
Analogue 4	Glu-Arg-Val-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 22
Analogue 5	Asp-Lys-Val-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 23
Analogue 6	Asp-Arg-Ala-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 24
Analogue 7	Asp-Arg-Val-Thr-Ile-His-Pro-Phe	SEQ ID NO: 25
Analogue 8	Asp-Arg-Val-Tyr-Leu-His-Pro-Phe	SEQ ID NO: 26
Analogue 9	Asp-Arg-Val-Tyr-Ile-Arg-Pro-Phe	SEQ ID NO: 27
Analogue 10	Asp-Arg-Val-Tyr-Ile-His-Ala-Phe	SEQ ID NO: 28
Analogue 11	Asp-Arg-Val-Tyr-Ile-His-Pro-Tyr	SEQ ID NO: 29
Analogue 12	Pro-Arg-Val-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 30
Analogue 13	Asp-Arg-Pro-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 31
Analogue 14	Asp-Arg-Val-Tyr(Po_3)$_2$-Ile-His-Pro-Phe	SEQ ID NO: 32
Analogue 15	Asp-Arg-norLeu-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 33
Analogue 16	Asp-Arg-Val-Tyr-norLeu-His-Pro-Phe	SEQ ID NO: 34
Analogue 17	Asp-Arg-Val-homoSer-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 35

The polypeptides of the instant invention may be synthesized by methods such as those set forth in J. M. Stewart and J. D. Young, Solid Phase Peptide Synthesis, 2nd ed., Pierce Chemical Co., Rockford, Ill. (1984) and J. Meienhofer, Hormonal Proteins and Peptides, Vol. 2, Academic Press, New York, (1973) for solid phase synthesis and E. Schroder and K. Lubke, The Peptides, Vol. 1, Academic Press, New York, (1965) for solution synthesis. The disclosures of the foregoing treatises are incorporated by reference herein.

In general, these methods involve the sequential addition of protected amino acids to a growing peptide chain (U.S. Patent No. 5,693,616, herein incorporated by reference in its entirety). Normally, either the amino or carboxyl group of the first amino acid and any reactive side chain group are protected. This protected amino acid

is then either attached to an inert solid support, or utilized in solution, and the next amino acid in the sequence, also suitably protected, is added under conditions amenable to formation of the amide linkage. After all the desired amino acids have been linked in the proper sequence, protecting groups and any solid support are removed to afford the crude polypeptide. The polypeptide is desalted and purified, preferably chromatographically, to yield the final product.

In one aspect of the present invention, a method of increasing *in vitro* and *ex vivo* hepatic cell proliferation by exposure to angiotensinogen, AI, AI analogues, and/or AI fragments and analogues thereof, AII analogues, AII fragments or analogues thereof, and/or AII AT_2 type 2 receptor agonists ("active agents"), is disclosed. Experimental conditions for the isolation, purification, *ex vivo* growth and *in-vivo* mobilization of hepatic stem cells and hepatocytes have been reported (Drakes et al., *J. Immunol.* 159:4268 (1997); Omori et al. *Hepatology* 26:720, (1997); U.S. Patent No. 4,914,032; U.S. Patent No. 5,227,158; all references incorporated by reference herein in their entirety).

In one embodiment of the invention, hepatocytes are isolated using procedures known in the art. (for example, see Free Radical Biology and Medicine 18:303-310). The isolated hepatocytes are resuspended in appropriate tissue culture medium to induce cell adherence. After 5-7 days in culture, the cells are treated with the active agents at various concentrations, preferably ranging from 0.1 ng/ml to 10 mg/ml. Cell proliferation is assessed at various time points during culture using methods well known in the art, including, but not limited to, measuring the rate of DNA synthesis according to the method of Nakamura and coworkers (Nakamura et al., *J. Biochem.* (Tokyo) 94:1029 (1982); Nakamura et al., *Biochem. Biophys. Res. Comm.* 122:1450

(1984)) Trypan blue dye exclusion/hemacytometer counting (Omori et al., *Hepatology* 26:720 (1997)), or flow cytometry (Drakes, *J. Immunol.* 159:4268 (1997); incorporated by reference herein in its entirety) to determine if the active agents have induced the proliferation of hepatocytes at a higher rate than control samples.

In another embodiment of the invention, hepatic stem cells are isolated from non-transplantable adult mammalian livers, particularly from the bile duct epithelium (Drakes et al., *J. Immunol.* 159:4268 (1997); Omori et al. *Hepatology* 26:720, (1997)). Hepatic stem cells are then selected for in these samples and cultured under appropriate growth conditions, in the presence of the active agents of the invention. Hepatic stem cell proliferation is assessed at various time points during culture as described above.

In a preferred embodiment, hepatic stem cells are isolated from adult mammalian livers by centrifugal elutriation of dispersed liver tissue (Hayner et al., *Cancer Res.* 44:332 (1984); Germain et al., *Cancer Res.* 45:673 (1985) Germain et al., *Cancer Res.* 48:368 (1988); Drakes et al., *J. Immunol.* 159:4268 (1997); Omori et al., *Hepatology* 26:720 (1997)). Briefly. adult mammalian liver is perfused *in situ* via the portal vein in two steps, first with Mg^{2+}- and Ca^{2+}-free Hank's balanced salt solution immediately followed by 0.1% pronase and 0.1% collagenase in buffered William's solution. Dispersed liver cells are then successively filtered on 240mm-pore and 70mm-pore nylon screens. The parenchymal cell fraction is obtained after multiple washings by centrifugation at 50 x *g* for 3 minutes. The nonparenchymal cell fraction is obtained by centrifugation at 200 x *g* for 10 minutes of the supernatant from which hepatocytes had been repeatedly eliminated by centrifugation. Following isolation, hepatic stem cells are suspended in standard culture medium, including but not limited to, RPMI-1640 (Gibco BRL, MD) and incubated in the presence of, preferably,

between about 0.1 ng/ml and about 10 mg/ml of the active agents of the invention. The cells are expanded for a period of between 8 and 21 days, then cellular proliferation is monitored by measuring the rate of DNA synthesis (Nakamura et al., 1982; Nakamura et al., 1984). Differentiation of hepatic stem cells to hepatocytes or bile ductule cells is detected by measuring the expression of typical differentiated hepatocyte markers including, but not limited to, albumin, transferrin, and alpha-1-antitrypsin, using standard methods well known in the art. These methods can include, but are not limited to, Northern blot analyses or reverse transcriptase-polymerase chain reaction (RT-PCR) with marker-specific DNA primers (Omori et al., *Hepatology* 25:1115 (1997); Drakes et al., *J. Immunol.* 159:4268 (1997); Omori et al., *Hepatology* 26:720 (1997)). Similarly, "self-renewal" of hepatic stem cells is assessed periodically by phase-contrast light microscopy and/or immunohistochemistry (Overturf et al., *Am. J. Pathol.* 151:5 (1997)).

In a further preferred embodiment, hepatic stem cells, bile ductule cells, or hepatocytes that have been cultured in the presence of the active agents are used for *ex vivo* liver-directed gene therapy (Alt and Cushman, *J. Hepatol.* 23:746 (1995)). Prior to *ex vivo* liver-directed gene therapy, the cells are rinsed to remove all traces of culture fluid, resuspended in an appropriate medium and then pelleted and rinsed several times. After the final rinse, the cells are resuspended at between 0.7×10^6 and 50×10^6 cells per ml in an appropriate medium and reinfused into a subject through intravenous infusions. Following reinfusion, hepatic cell proliferation is assessed by the methods discussed above.

In one embodiment, assessment of the *in vivo* proliferative effect of the active agents of the invention on hepatic cells is performed by histochemical evaluations of

the liver including, but not limited to, hepatocytic parenchyma, periportal parenchyma, biliary epithelium, hyperplastic ducts within the periportal zone, arborizing ductules, and the portal spaces. In a preferred embodiment, *in vivo* proliferation of hepatic cells is assessed by reactivity to an antibody directed against a protein known to be present in higher concentrations in proliferating cells than in non-proliferating cells, such as proliferating cell nuclear antigen (PCNA or cyclin; Zymed Laboratories) (Rodgers et al., *J. Burn Care Rehabil.* 18:381-388 (1997); incorporated herein by reference in its entirety).

In another aspect of the present invention the active agents are used to increase *in vivo* hepatic cell proliferation. For use in increasing the proliferation of hepatic cells, the active agents may be administered by any suitable route, including orally, parenterally, by inhalation spray, rectally, transdermally, or topically in dosage unit formulations containing conventional pharmaceutically acceptable carriers, adjuvants, and vehicles. The term parenteral as used herein includes, subcutaneous, intravenous, intraarterial, intramuscular, intrasternal, intratendinous, intraspinal, intracranial, intrathoracic, infusion techniques or intraperitoneally.

The active agents may be made up in a solid form (including granules, powders or suppositories) or in a liquid form (*e.g.*, solutions, suspensions, or emulsions) and may be subjected to conventional pharmaceutical operations such as sterilization and/or may contain conventional adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers, buffers etc.

While the active agents of the invention can be administered as the sole active pharmaceutical agent, they can also be used in combination with one or more other compounds. When administered as a combination, the active agents and other

compounds can be formulated as separate compositions that are given at the same time or different times, or the active agents and other compounds can be given as a single composition.

For administration, the active agents are ordinarily combined with one or more adjuvants appropriate for the indicated route of administration. The active agents may be admixed with lactose, sucrose, starch powder, cellulose esters of alkanoic acids, stearic acid, talc, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulphuric acids, acacia, gelatin, sodium alginate, polyvinylpyrrolidine, and/or polyvinyl alcohol, and tableted or encapsulated for conventional administration. Alternatively, the active agents of this invention may be dissolved in saline, water, polyethylene glycol, propylene glycol, carboxymethyl cellulose colloidal solutions, ethanol, corn oil, peanut oil, cottonseed oil, sesame oil, tragacanth gum, and/or various buffers. Other adjuvants and modes of administration are well known in the pharmaceutical art. The carrier or diluent may include time delay material, such as glyceryl monostearate or glyceryl distearate alone or with a wax, or other materials well known in the art.

Formulations suitable for topical administration include liquid or semi-liquid preparations suitable for penetration through the skin (*e.g.*, liniments, lotions, ointments, creams, or pastes) and drops suitable for administration to the eye, ear, or nose.

The dosage regimen for increasing *in vivo* proliferation of hepatic cells with the active agents of the invention is based on a variety of factors, including the type of injury, the age, weight, sex, medical condition of the individual, the severity of the condition, the route of administration, and the particular compound employed. Thus,

the dosage regimen may vary widely, but can be determined routinely by a physician using standard methods. Dosage levels of the order of between 0.1 ng/kg and 10 mg/kg of the active agents per body weight are useful for all methods of use disclosed herein.

The treatment regime will vary depending on the condition being treated, based on a variety of factors, including the type of injury, the age, weight, sex, medical condition of the individual, the severity of the condition, the route of administration, and the particular compound employed.

In a preferred embodiment of the present invention, the active agents are administered parenterally. A suitable parenteral dose of the active agents is preferably between about 0.1 ng/kg and about 10 mg/kg administered twice daily. For parenteral administration, the active agent may comprise from 0.001% to 10% w/w, *e.g.*, from 1% to 2% by weight of the formulation, although it may comprise as much as 10% w/w, but preferably not more than 5% w/w, and more preferably from 0.1% to 1% of the formulation.

In another aspect of the present invention, an improved cell culture medium is provided for the proliferation of hepatic cells, wherein the improvement comprises addition to the cell culture medium of an effective amount of the active agents of the invention. Any cell culture media that can support the growth of hepatic cells can be used with the present invention. Such cell culture media include, but are not limited to Basal Media Eagle, Dulbecco's Modified Eagle Medium, Iscove's Modified Dulbecco's Medium, McCoy's Medium, Minimum Essential Medium, F-10 Nutrient Mixtures, Opti-MEM® Reduced-Serum Medium, and RPMI Medium, or combinations thereof.

The improved cell culture medium can be supplied in either a concentrated (ie: 10X) or non-concentrated form, and may be supplied as a liquid, a powder, or a

lyophilizate. The cell culture may be either chemically defined, or may contain a serum supplement. Culture media and serum supplements are commercially available from many sources, such as GIBCO BRL (Gaithersburg, MD) and Sigma (St. Louis, MO)

In a further aspect, the present invention provides kits for the propagation of hepatic cells, wherein the kits an effective amount of the active agents of the invention, and a set of instructions for culturing the cells.

In a preferred embodiment, the kit further comprises cell culture media. Any cell culture media that can support the growth of hepatic cells can be used with the present invention. Examples of such cell culture media are described above.

The improved cell culture medium can be supplied in either a concentrated (ie: 10X) or non-concentrated form, and may be supplied as a liquid, a powder, or a lyophilizate. The cell culture may be either chemically defined, or may contain a serum supplement.

In another preferred embodiment, the kit of the present invention further comprises a sterile container. The sterile container can comprise either a sealed container, such as a cell culture flask, a roller bottle, or a centrifuge tube, or a non-sealed container, such as a cell culture plate or microtiter plate (Nunc; Naperville, IL).

In a further preferred embodiment, the kit further comprises an antibiotic supplement for inclusion in the reconstituted cell growth medium. Examples of appropriate antibiotic supplements include, but are not limited to actimonycin D, Fungizone®, kanamycin, neomycin, nystatin, penicillin, streptomycin, or combinations thereof (GIBCO).

The present invention may be better understood with reference to the accompanying examples that are intended for purposes of illustration only and should

not be construed to limit the scope of the invention, as defined by the claims appended hereto.

Example 1. AII and AII analogues and fragments increase hepatocyte proliferation

Hepatocytes were isolated from rabbits using the procedure of Morel et al. (Free Radical Biology and Medicine 18:303-310). Briefly, the liver was minced and placed in culture with 0.02% Type VIII collagenase (Sigma, St. Louis, MO) in RPM1640 overnight at 37° C. Thereafter, the cells were collected in RPMI 1640 medium containing 1 mg/ml bovine serum albumin and 5 μg/ml bovine insulin. The cell suspension was filtered through sterile gauze and allowed to sediment for 20 minutes to remove cellular debris and blood. The cells were then washed three times by centrifugation at 1200 rpm. Viability was assessed by Trypan blue exclusion. The hepatocytes were resuspended in 75% Eagle's minimal essential medium/25% Medium 199 (Gibco BRL) buffered with sodium bicarbonate and supplemented with 10% fetal calf serum containing 50 μg/ml streptomycin, 7.5 IU/ml penicillin, 5 μg/ml insulin, and 1 mg/ml bovine serum albumin at 1 x 10^6 cells/ml. The medium was changed after 3-4 hours of adherence and was changed again after 24 hours. The cells were cultured for 5-7 days prior to use in the following studies.

After 5-7 days in culture, the cells were detached from the tissue culture plastic with 0.05% trypsin-EDTA (Gibco-BRL Products) at 37°C for 10 minutes. The cells were washed one time with sterile phosphate buffered saline (pH 7.2) and adjusted to a concentration of 200 cells/ml. Two hundred μl of this suspension was aliquoted into wells of 96 well plates and allowed to attach to the tissue culture plastic. After adherence, AII, AII analogues and AII fragments were added to the wells in duplicate

to a final concentration of 10 μg/ml. The identity of the AII analogues and fragments is shown in Table 1.

Table 1. Designation for Analogues

Name	Abbreviation	Sequence	SEQ ID NO:
GSD 28	Ile^8-AII	DRVYIHPI	SEQ ID NO: 38
GSD 24B	Pro^3-AII	DRPYIHPF	SEQ ID NO:31
GSD 22A	Ala^4-AIII	RVYAHPF	SEQ ID NO:18
AII(1-7)		DRVYIHP	SEQ ID NO:4
AII		DRVYIHPF	SEQ ID NO. 1

On days 1, 2, or 3 after addition of the peptides, the medium was removed, the cells were stained with Geimsa stain to identify nuclei, and the number of nucleated cells present in each well was assessed microscopically. The results are shown in Figure 1. These data demonstrate that AII, AII(1-7) and the AII analogues and fragments significantly increased the number of hepatocytes per well relative to a control.

The present invention, by providing methods for enhanced proliferation of hepatic cells, will greatly increase the clinical uses of hepatic cells for liver regeneration after resection of hepatocarcinomas and in other hepatic disorders where a more rapid regeneration of the liver is desirable. This is true both for increased "self-renewal", which will provide a larger supply of hepatic stem cells capable of generating additional hepatocytes, epithelial cells, and/or exocrine pancreatic cells and for proliferation with differentiation, which will provide a larger supply of epithelial cells, hepatocytes, or exocrine pancreatic cells, for use in liver regeneration.

The method of the present invention also increases the potential utility of hepatic cells as vehicles for gene therapy in hepatic system disorders by more

efficiently providing a large number of such cells for transfection, and also by providing a more efficient means to rapidly expand transfected hepatic cells.

The present invention is not limited by the aforementioned particular preferred embodiments. It will occur to those ordinarily skilled in the art that various modifications may be made to the disclosed preferred embodiments without diverting from the concept of the invention. All such modifications are intended to be within the scope of the present invention.

We claim:

1. A method for promoting hepatic cell proliferation comprising contacting hepatic cells with an amount effective to promote proliferation of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group,

R^A is suitably selected from Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, Me^2Gly, Pro, Bet, Glu(NH_2), Gly, Asp(NH_2) and Suc,

R^B is suitably selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr(PO_3)$_2$, Thr, Ser, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group,

and wherein the active agent is not AII.

2. The method of claim 1 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36; SEQ ID NO:37, and SEQ ID NO:38.

3. The method of claim 1 wherein the concentration of active agent is between about 0.1 ng/kg and about 10.0 mg/kg.

4. A method for promoting hepatic cell proliferation comprising contacting hepatic cells with an amount effective to promote proliferation of an active agent comprising a sequence of the following general formula:

R1-Arg-R2-R3-R4-His-Pro-R5

wherein R1 is selected from the group consisting of H and Asp;

R2 is selected from the group consisting of Val and Pro;

R3 is selected from the group consisting of Tyr and $Tyr(PO_3)_2$;

R4 is selected from the group consisting of Ala, Ile, Leu, and norLeu; and

R5 is Phe, Ile, or is absent,

and wherein the active agent is not AII.

5. The method of claim 4 wherein the active agent is selected from the group consisting of SEQ ID NO:4, SEQ ID NO:18, SEQ ID NO:26, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:34, and SEQ ID NO:38.

6. The method of claim 4 wherein the concentration of active agent is between about 0.1 ng/kg and about 10.0 mg/kg.

7. An improved cell culture medium for promotion of hepatic cell proliferation, wherein the improvement comprises addition to the cell culture medium an amount effective to increase proliferation of hepatic cells of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group,

R^A is suitably selected from Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, Me^2Gly, Pro, Bet, Glu(NH_2), Gly, Asp(NH_2) and Suc,

R^B is suitably selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr(PO_3)$_2$, Thr, Ser, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group,

and wherein the active agent is not AII.

8. The improved cell culture medium of claim 7 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36; SEQ ID NO:37, and SEQ ID NO:38.

9. The improved cell culture medium of claim 7 wherein the concentration of active agent is between about 0.1 ng/ml and about 10.0 mg/ml.

10. An improved cell culture medium for promotion of hepatic cell proliferation, wherein the improvement comprises addition to the cell culture medium an amount effective to increase proliferation of hepatic cells of at least one active agent comprising a sequence of the following general formula:

R1-Arg-R2-R3-R4-His-Pro-R5

wherein R1 is selected from the group consisting of H and Asp;

R2 is selected from the group consisting of Val and Pro;

R3 is selected from the group consisting of Tyr and $Tyr(PO_3)_2$;

R4 is selected from the group consisting of Ala, Ile, Leu, and norLeu; and

R5 is Phe, Ile, or is absent,

and wherein the active agent is not AII.

11. The improved cell culture medium of claim 10 wherein the active agent is selected from the group consisting of SEQ ID NO:4, SEQ ID NO:18, SEQ ID NO:26, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:34, and SEQ ID NO:38.

12. The improved cell culture medium of claim 10 wherein the concentration of active agent is between about 0.1 ng/ml and about 10.0 mg/ml.

13. A kit for promoting hepatic cell proliferation comprising:

(a) an amount effective to promote hepatic cell proliferation of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group,

R^A is suitably selected from Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, Me^2Gly, Pro, Bet, Glu(NH_2), Gly, Asp(NH_2) and Suc,

R^B is suitably selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr(PO_3)$_2$, Thr, Ser, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group,

wherein the active agent is not AII; and

(b) instructions for using the amount effective of active agent to promote hepatic cell proliferation.

14. The kit of claim 13 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36; SEQ ID NO:37, and SEQ ID NO:38.

15. The kit of claim 13 wherein the concentration of active agent is between about 0.1 ng/ml and about 10.0 mg/ml.

16. A kit for promoting hepatic cell proliferation comprising an amount effective to promote hepatic cell proliferation of an active agent comprising a sequence of the following general formula:

R1-Arg-R2-R3-R4-His-Pro-R5

wherein R1 is selected from the group consisting of H and Asp;

R2 is selected from the group consisting of Val and Pro;

R3 is selected from the group consisting of Tyr and $Tyr(PO_3)_2$;

R4 is selected from the group consisting of Ala, Ile, Leu, and norLeu; and

R5 is Phe, Ile, or is absent,

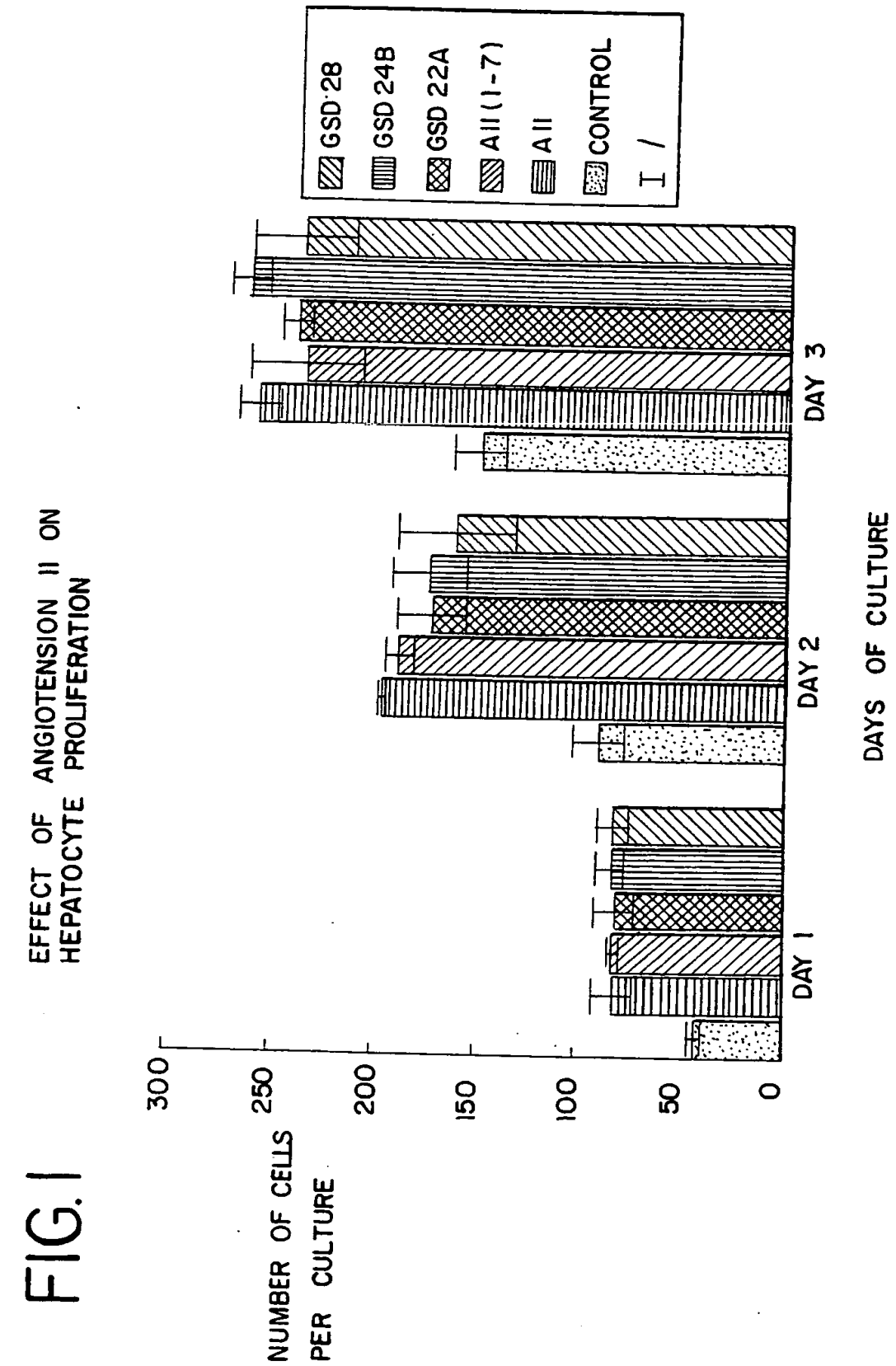
and wherein the active agent is not AII; and

(b) instructions for using the amount effective of active agent to promote hepatic cell proliferation.

17. The kit of claim 16 wherein the active agent is selected from the group consisting of SEQ ID NO:4, SEQ ID NO:18, SEQ ID NO:26, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:34, and SEQ ID NO:38.

18. The kit of claim 16 wherein the concentration of active agent is between about 0.1 ng/ml and about 10.0 mg/ml.

FIG. 1



SEQUENCE LISTING

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Arg Val Tyr Xaa His Pro Phe

1 5

<210> 14

<211> 3

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII(6-8)

<400> 14

His Pro Phe

1

<210> 15

<211> 5

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII(4-8)

<400> 15

Tyr Ile His Pro Phe

1 5

<210> 16

<211> 7

<212> PRT

<213> Artificial Sequence

<220>

<221> Xaa at position 1 can be Hydrogen, Arg, Lys, Ala, Orn, Ser(Acetylated), MeGly, D-Arg, or D-Lys;

Xaa at position 2 can be Val, Ala, Leu, Nle, Ile, Gly, Pro, Aib, Acp, or Tyr; Xaa at position 4 can be Ile, Ala, Leu, Nle, Val, or Gly

<222> 1-4

<223> Description of Artificial Sequence:AII analogue class

<400> 16

Xaa Xaa Tyr Xaa His Pro Phe

1 5

<210> 17

<211> 7

<212> PRT

<213> Artificial Sequence

<220>

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<400> 17

Arg Val Tyr Gly His Pro Phe

1 5

<210> 18

<211> 7

<212> PRT

<213> Artificial Sequence

<220>

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<400> 18

Arg Val Tyr Ala His Pro Phe

1 5

<210> 19

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII analogue i

<400> 19

Asp Arg Val Tyr Val His Pro Phe

1 5

<210> 20

<211> 8

<212> PRT

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<220>

<223> Description of Artificial Sequence:AII Analogue 2

<400> 20

Asn Arg Val Tyr Val His Pro Phe

1 5

<210> 21

<211> 11

<212> PRT

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<220>

<223> Description of Artificial Sequence:AII Analogue 3

<400> 21

Ala Pro Gly Asp Arg Ile Tyr Val His Pro Phe

1 5 10

<210> 22

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII Analogue 4

<400> 22

Glu Arg Val Tyr Ile His Pro Phe

1 5

<210> 23

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII Analogue 5

<400> 23

Asp Lys Val Tyr Ile His Pro Phe

1 5

<210> 24

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII Analogue 6

<400> 24

Asp Arg Ala Tyr Ile His Pro Phe

1 5

<210> 25

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII Analoge 7

<400> 25

Asp Arg Val Thr Ile His Pro Phe

1 5

<210> 26

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII Analogue 8

<400> 26

Asp Arg Val Tyr Leu His Pro Phe

1 5

<210> 27

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII Analogue 9

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Asp Arg Val Tyr Ile Arg Pro Phe

1 5

<210> 28

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII Analogue 10

<400> 28

Asp Arg Val Tyr Ile His Ala Phe

1 5

<210> 29

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII Analogue 11

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Asp Arg Val Tyr Ile His Pro Tyr

1 5

<210> 30

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:AII Analogue 12

<400> 30

Pro Arg Val Tyr Ile His Pro Phe

1 5

<210> 31

<211> 8

<212> PRT

<213> Artificial Sequence

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<400> 31

Asp Arg Pro Tyr Ile His Pro Phe

1 5

<210> 32

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<221> PHOSPHORYLATION

<222> 4

<223> Description of Artificial Sequence:AII Analogue 14

<400> 32

Asp Arg Val Tyr Ile His Pro Phe

1 5

<210> 33

<211> 8

<212> PRT

<213> Artificial Sequence

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<221> Xaa at position 3 is Nle

<222> 3

<223> Description of Artificial Sequence:AII Analogue 15

<400> 33

Asp Arg Xaa Tyr Ile His Pro Phe

1 5

<210> 34

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<221> Xaa at position 5 is Nle

<222> 5

<223> Description of Artificial Sequence:AII Analogue 16

<400> 34

Asp Arg Val Tyr Xaa His Pro Phe

1 5

<210> 35

<211> 9

<212> PRT

<213> Artificial Sequence

<220>

<221> homo Ser

<222> 4

<223> Description of Artificial Sequence:AII Analogue 17

<400> 35

Asp Arg Val Ser Tyr Ile His Pro Phe

1 5

<210> 36

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:p-aminophenylalanine 6 AII

<400> 36

Asp Arg Val Tyr Ile Xaa Pro Phe

1 5

<210> 37

<211> 10

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:angiotensin I

<400> 37

Asp Arg Val Tyr Ile His Pro Phe His Leu

1 5 10

<210> 38

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial

Sequence:GSD28: Ile5-AII

<400> 38

Asp Arg Val Tyr Ile His Pro Ile

1 5

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(54) Title: METHOD OF PROMOTING HEPATIC CELL PROLIFERATION

(57) Abstract

The present invention provides methods, improved cell culture medium and kits for promoting hepatic cell proliferation and differentiation by growth in the presence of angiotensinogen, AI, AI anoalogues, and/or AI fragments and analogues thereof, AII analogues, AII fragments or analogues thereof, and/or AII AT_2 type 2 receptor agonists, either alone or in combination with other growth factors and cytokines.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/02618

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/08 C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE CHEMABS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US DAJANI, OLAV F. ET AL: "Growth-promoting effects of Ca2+-mobilizing agents in hepatocytes: lack of correlation between the acute activation of phosphoinositide-specific phospholipase C and the stimulation of DNA synthesis by angiotensin II, vasopressin, norepinephrine, and prostaglandin F2.alpha." retrieved from STN Database accession no. 125:213302 HCA XP002111087 abstract & J. CELL. PHYSIOL. (1996), 168(3), 608-617 , --- -/--	1,2,7,13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 August 1999	12/08/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/02618

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 14858 A (UNIVERSITY OF SOUTHERN CALIFORNIA) 23 May 1996 (1996-05-23) the whole document	1-18
A	WO 96 39164 A (THE UNIVERSITY OF SOUTHERN CALIFORNIA) 12 December 1996 (1996-12-12) the whole document	1-18
P,A	WO 98 32457 A (UNIVERSITY OF SOUTHERN CALIFORNIA) 30 July 1998 (1998-07-30) the whole document	1-18

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/02618

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 1-6 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INT ATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No
PCT/US 99/02618

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9614858 A	23-05-1996	US 5629292 A	13-05-1997
		AU 4364896 A	06-06-1996
		CA 2205092 A	23-05-1996
		CN 1172434 A	04-02-1998
		EP 0792158 A	03-09-1997
WO 9639164 A	12-12-1996	US 5834432 A	10-11-1998
		AU 5990796 A	24-12-1996
		CA 2221730 A	12-12-1996
		EP 0828505 A	18-03-1998
WO 9832457 A	30-07-1998	AU 6648598 A	18-08-1998
		WO 9926644 A	03-06-1999